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Review Article

Quantitative measures for redox signaling

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ABSTRACT

Redox signaling is now recognized as an important regulatory mechanism for a number of cellular processes including the antioxidant response, phosphokinase signal transduction and redox metabolism. While there has been considerable progress in identifying the cellular machinery involved in redox signaling, quantitative measures of redox signals have been lacking, limiting efforts aimed at understanding and comparing redox signaling under normoxic and pathogenic conditions. Here we have outlined some of the accepted principles for redox signaling, including the description of hydrogen peroxide as a signaling molecule and the role of kinetics in conferring specificity to these signaling events. Based on these principles, we then develop a working definition for redox signaling and review a number of quantitative methods that have been employed to describe signaling in other systems. Using computational modeling and published data, we show how time- and concentration- dependent analyses, in particular, could be used to quantitatively describe redox signaling and therefore provide important insights into the functional organization of redox networks. Finally, we consider some of the key challenges with implementing these methods.

Contents

1. Introduction

Cellular signaling involves a set of important, and usually interconnected physiological processes that allow cells to adapt and respond to changes in their internal and external states [1]. The

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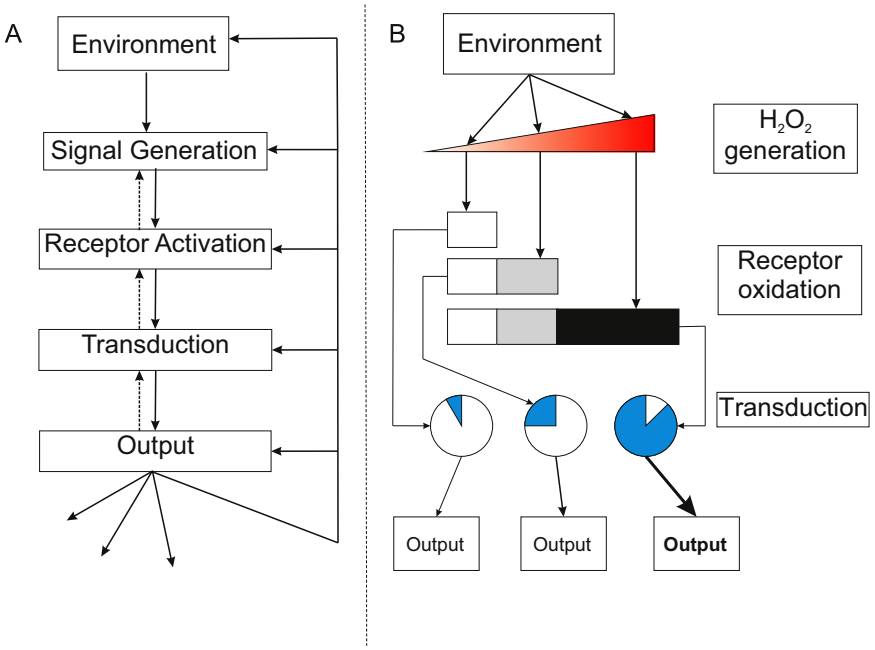

Fig. 1. A comparison of canonical signaling and redox signaling processes. Depending on the specific context, canonical signaling processes involve the production of a signal which is sensed by a receptor which in turn activates transduction machinery resulting in a cellular output (A). The components that make up the steps of this signaling process may be combined into single steps or may involve multiple molecular activation processes. In redox signaling (B), hydrogen peroxide is believed to be the predominant redox signaling molecule and its production can be stimulated by endogenous and exogenous environmental stimuli. At low concentrations, hydrogen peroxide is expected to activate only a subset of the proteome resulting in specific outputs but at higher concentrations a wider range of targets may be oxidized resulting in progressively non-specific outputs.

basic conceptual underpinnings of the signaling process have been well established beginning with the intra- or extracellular perturbations that result in a signal which activates a specific receptor (s), which in turn activates downstream transduction machinery (Fig. 1A). Depending on the pathway, the signal generation, receptor activation and transduction processes may be distinct or may be combined into the same set of signaling components. The transduced signal ultimately results in outputs that can affect global and/or specific physiological processes including the signaling process itself (Fig. 1A). The molecular state (*e.g.* phosphorylation) and activity changes in the transduction and output machinery are normally used for characterizing signal propagation, although crucially, signal transmission at each step in this process is balanced by signal deactivation mechanisms which set thresholds for signal propagation [2,3].

Quantitative approaches have been extremely useful in elucidating the dynamics of signaling and have often yielded surprising insights on the design principles underlying signal transduction. For example, analysis of the kinetic structure of mitogen-activated protein kinase (MAPK) pathways revealed the basis for ultrasensitive signal responses [4] and a host of other signaling motifs that could not have been predicted by studying the MAPKs in isolation [5]. Further, using quantitative measures of signal transduction [2], it was shown that control of signal duration within MAPK pathways lay with the deactivating phosphatases and not kinases in these systems [3]. Similarly, studies on genetic circuits have revealed how motifs such as negative autoregulation can affect the transcription rate of target genes and therefore the output of a given signaling event [6,7].

Redox signaling is now emerging as an important signaling process and, together with clinical studies in the redox biology field, has changed perceptions of the role played by reactive oxygen species (ROS) such as hydrogen peroxide in cell physiology. The use of the term reactive oxygen species (ROS) is contentious and we will therefore only use it if the identity of the oxidant(s) in a physiological process is unknown [8]. Like most other ROS, hydrogen peroxide has generally been viewed as a toxic agent, although its mitogenic properties have long been known [9] and there is a growing appreciation that it is an important mediator of signaling events [10–15]. In fact, dampening intrinsic ROS levels by exogenous antioxidants may actually be detrimental in some cases [16–19]. While the mechanistic details underlying redox signaling are continually being uncovered, most studies have not quantitatively probed the nature of the redox signal. The aim of this paper is to review some of the quantitative measures for signaling and determine whether they are applicable for redox signaling, as these measures would allow for a better framework to understand redox signal transduction and its role in pathophysiological processes. As a first step we have considered some of the principles that have emerged from previous studies.

2. Emerging principles from redox signaling studies

Data from kinetic, genetic and proteomic studies have provided insights into some of the basic principles underlying thiol-based redox signaling which have been described extensively in several excellent reviews [10,11,20–27]. For clarity, these principles will be briefly described below.

2.1. Cellular context is important for redox signaling

Determining a consistent quantitative framework for redox signaling in all organisms is difficult for a number of reasons. In contrast to other signaling systems such as phosphokinase signaling, hydrogen peroxide can non-specifically oxidize a broad range of molecular targets at sufficiently high concentrations and can therefore trigger a wider set of responses than canonical signaling systems (Fig. 1B, [9,28]). The distinction, if any, between redox signaling and the oxidative stress response is therefore challenging as these responses utilize a common set of machinery. Further, depending on their niches, different cells appear to have

distinct relationships to hydrogen peroxide. For example, many bacterial cells contain proteins and co-factors that are extremely sensitive to hydrogen peroxide and therefore maintain intracellular hydrogen peroxide levels within the nanomolar range [28–30]; hydrogen peroxide and other oxidant-dependent signaling in these cells is used largely to prime the oxidative stress defense machinery [31,32]. On the other hand, mammalian cells can tolerate larger hydrogen peroxide production fluxes and also specifically generate hydrogen peroxide to enhance MAPK and insulin signaling. Nonetheless, even in these cells, growth arrest and cell death can result from higher intracellular hydrogen peroxide levels ($>1\ \mu M$) [28,33–38]. In addition, there appear to be considerable variations in the concentrations of redoxins in different mammalian tissues [39] and redox signaling may therefore show tissue-specific differences even within the same organism.

2.2. Hydrogen peroxide is a redox signaling molecule

Of all the ROS, hydrogen peroxide appears to have the most favorable properties for mediating redox-signaling events. Hydrogen peroxide is specifically transported across membranes by aquaporins [40,41] and intracellular hydrogen peroxide levels appear to be homeostatically regulated across all organisms. All cells generate hydrogen peroxide through metabolic activity [29,30,42,43], but its production can also be specifically catalyzed by NADPH oxidases and cytochrome P450 enzymes in mammalian cells [24]. In plant cells, hydrogen peroxide can be produced by NADPH-dependent oxidases [44] and plays a central role in a large number of signaling processes including the generation of hydrogen peroxide waves used in cell-to-cell communication [45,46].

While hydrogen peroxide is relatively stable and abundant (half-life $=10^{-3}-10^{-5}$ s) [22,47,48] compared to oxidants such as the hydroxyl radical (half-life $=10^{-9}$ s) [38], it can react rapidly with specialist peroxidases and catalases with rate constants ranging from 10^4 to $10^8\ M^{-1}\ s^{-1}$, while the rate constants for other biomolecules can range from as low as $0.87\ M^{-1}\ s^{-1}$ for small thiols such as glutathione [49] to as high as $10^4\ M^{-1}\ s^{-1}$ for metal co-factor containing proteins [30,32,50,51]. Thus, only a subset of the proteome would be expected to react and transmit redox signals under the limiting hydrogen peroxide concentrations prevailing in most cells [23] conferring specificity to redox signaling events. Correspondingly only a fraction of the total proteome has been identified as targets for hydrogen peroxide-dependent oxidation [38,52]. However, under severe oxidative stress conditions, a larger range of targets can be oxidized resulting in additional redox modifications and redox signaling mechanisms such as glutathionylation [53–56], leading to broader, non-specific responses and ultimately toxicity and cell death [12,30,44,48,50,57]. It should be noted that the glutathionylation/deglutathionylation cycle also regulates the activity of some proteins under normoxic conditions. However, as glutathionylation largely depends on the formation of activated thiol derivatives, this mechanism is more prevalent under oxidative stress conditions [55,58]. An additional factor contributing to specificity in hydrogen peroxide signaling is its effective intracellular diffusion distance which, depending on cell size and composition, can confine hydrogen peroxide generation and signaling to specific intracellular regions or microdomains [10,59].

2.3. Redox signaling depends on primarily on kinetic and not thermodynamic considerations

Hydrogen peroxide has a relatively high redox potential [22,47] and can in principle accept electrons from most biologically relevant redox couples including the glutathione/oxidized glutathione (GSH/GSSG), cysteine/cystine and thioredoxin redox couples. Much of the redox biology literature had therefore focused on how changes in the redox potentials of these couples correlated with distinct cell and tissue physiological states such as differentiation capacity [60–64]. However, a number of serious limitations with this approach have been recognized, not least of which is that the mechanism responsible for these correlations was largely undefined [65,66]. It was therefore unclear whether the changes in the redox potential of these redox couples represented redox signal-dependent physiological changes in these cells or were merely indicators of underlying kinetic processes. While there are limitations to using redox potentials alone to describe redox signaling [51,66], many thiol-based redox systems depend on NADPH oxidation to provide the thermodynamic driving force for reduction. Uncoupling such redox systems from this metabolite therefore converts cytoplasmic reductants such as thioredoxin into oxidants [67]. Moreover, under oxidative stress conditions the flux from glycolysis is redirected to NADPH reduction [68]. These results argue that the redox signaling machinery is dependent on thermodynamic coupling to highly electronegative redox pairs such as NADP/NADPH and can therefore be affected by the broader metabolic state of the cell.

There has been a growing consensus that redox signaling is regulated by the kinetics of the putative signaling machinery with respect to hydrogen peroxide [12,57]. In principle, the relevant species concentrations and kinetic parameters for hydrogen peroxide reduction could consequently be useful guides for identifying or excluding molecules that can act as genuine redox signaling components. Indeed, computational simulations and wet-lab studies have revealed that the primary reactants of hydrogen peroxide are specialist hydrogen peroxide scavengers with second-order rate constants greater than or equal to $10^4\ M^{-1}s^{-1}$ [10]. However, as will be described below, localized hydrogen peroxide accumulation could result in the oxidation of targets that theoretically should have been out-competed by specialist hydrogen peroxide scavengers.

Aerobically growing cells employ a host of such scavenging enzymes that could regulate redox signaling by hydrogen peroxide, including catalases and peroxidases. Catalases use a disproportion cycle to reduce hydrogen peroxide and therefore become more effective as the intracellular hydrogen peroxide concentration increases, while peroxidases use an exogenous electron donor to complete their catalytic cycles [30]. Under normoxic conditions peroxidases are considered to be more effective than catalases, but as their reduction is often a rate-limiting step in the peroxidase catalytic cycle, their activity can be affected by availability of their cognate reductants [30]. Based on their hydrogen peroxide reduction kinetics, glutathione peroxidases and peroxiredoxins are among the best candidates as hydrogen peroxide sensors and redox signal transducers [10,25,69]. Glutathione peroxidases react rapidly with hydroperoxides ($10^3-10^7\ M^{-1}\ s^{-1}$) and are in turn reduced by glutathione in an enzyme-substitution mechanism [69,70]. As these peroxidases are present at significantly lower intracellular concentrations than peroxiredoxins, they are presumably hydrogen peroxide scavengers rather than specialist redox signal transducers [25]. Nonetheless glutathione peroxide dysregulation has been associated with diseases such as obesity [71] suggesting that these peroxidases can play a role in other signaling systems.

Peroxiredoxins also react rapidly with hydrogen peroxide with rate constants as high as $10^8\ M^{-1}\ s^{-1}$, are present at relatively high intracellular concentrations and are the primary sensors for hydrogen peroxide in most cells [10,72,73]. There are three major classes of peroxiredoxins, typical 2-Cys, atypical 2-Cys and 1-Cys peroxiredoxins, although based on functional site profiling, peroxiredoxins may consist of six sub-families, some of which can be further-divided into additional groups [74]. Upon hydrogen

peroxide reduction, an active site peroxidatic cysteine is oxidized to a sulfenic acid (SOH), which then condenses into an inter- or intra-molecular disulfide bond for typical and atypical 2-Cys peroxiredoxins, respectively. For most peroxiredoxins, this disulfide bond is reduced by the thioredoxin system although for some peroxiredoxins this bond is reduced by the GSH/glutaredoxin system [75–77] or by both thioredoxin and GSH [78]. In the alkyl hydroperoxidase system (AhpCF) of Gram-negative bacteria, the AhpC peroxiredoxin is directly reduced by the flavoprotein AhpF using NADH as a reducing substrate [79,80]. For 1-Cys peroxiredoxins, the catalytic cycle also appears to involve the formation of a sulfenic acid which can be reduced by ascorbic acid or DTT *in vitro* [81], by π glutathione-S-transferases in mammalian cells [82] or can form a disulfide with glutathione which is then reduced [83].

In addition to their rapid hydrogen peroxide reduction kinetics, 2-Cys peroxiredoxins have a number of intriguing structural and catalytic features that are relevant to redox signaling [84,85]. Under reducing conditions and when their concentrations are in the micromolar range [26], 2-Cys peroxiredoxins form head-to-tail homodimers that assemble into ring-like decamers [86]. During catalysis, oxidation and subsequent disulfide bond formation of the active site cysteine residues weakens the dimer-dimer interfaces and the decamer dissociates into disulfide-linked dimers which are consequently reduced [84,85]. However, in the presence of high hydrogen peroxide concentrations, the peroxidatic sulfenic intermediate can be further oxidized to a sulfinic acid and these hyperoxidized peroxiredoxins assemble high molecular weight oligomers with chaperone functions but with reduced peroxidase and consequently thioredoxin-oxidation activities [87]. The hyperoxidized peroxiredoxin state is reversed by sulfiredoxins in an ATP-dependent process [88,89] and curiously, this sensitivity to oxidation is found more commonly, but not exclusively [90], in mammalian peroxiredoxins suggesting this mode of regulation is not universal [91].

Peroxiredoxins have been proposed to transduce redox signaling through two distinct but not necessarily exclusive models, the 'floodgate' and 'signal peroxidase' models. In the floodgate model, peroxiredoxins are inactivated by the hyperoxidation allowing for the localized accumulation of hydrogen peroxide, which can then oxidize signaling proteins in a reaction that is usually outcompeted by peroxiredoxins under standard conditions [92]. While variations in hyperoxidized peroxiredoxin levels constitute a recognized indicator of circadian rhythms [93–96], it has been shown that hydrogen peroxide production during mitogen-signaling in mammalian cells and over the lifespan of *Caenorhabditis elegans* was not sufficient to over-oxidize peroxiredoxins [97–100], and therefore additional mechanisms for peroxide inactivation were sought. Mammalian Src and mammalian sterile twenty (Mst) kinases whose activities can stimulate or are stimulated by hydrogen peroxide production can phosphorylate peroxiredoxins, reducing their peroxidase activity and facilitating the transient accumulation of hydrogen peroxide [59,101]. In contrast, Ser-32 phosphorylation of Prx1 in RPMI7591 melanoma cells increased the hydrogen peroxide degradation capacity of the peroxiredoxins [102], which hints at additional regulatory features within this mode of redox signaling and emphasizes the sometimes cell-specific nature of redox signaling events. In addition to phosphorylation, glutathionylation, acetylation and nitrosylation can also regulate peroxiredoxin activity and conformation [26]. However, a significant limitation with the floodgate model is that it is unclear how signaling proteins can outcompete glutathione peroxidases or even glutathione for hydrogen peroxide generated during the signaling event [25,57].

The signal peroxidase model differs from the floodgate model in that the peroxiredoxins and signaling proteins do not compete for hydrogen peroxide. Rather, peroxiredoxins themselves act as hydrogen peroxide sensors and transmit changes in their oxidation state by thiol-disulfide exchange [15,98,103]. Both *in vitro* and *in vivo* data in yeast and mammalian systems have shown that the oxidation of certain transcription factors and kinases was dependent on peroxiredoxin oxidation. For example, oxidation of the baker's yeast Gpx3 peroxidatic cysteine to a sulfinic acid leads to thiol-disulfide exchange with the Yap1 transcription factor in the presence of Ypb1 [104–106], masking the Yap1 nuclear export signal and allowing it to accumulate in the nucleus [107,108]. Thus, in the signal peroxidase model, thiol-disulfide relays are used to transduce redox signals. Only a fraction of the peroxiredoxin sensor and its target protein(s) may participate in these redox relays [103] and their interaction is facilitated by adaptor proteins or redox microdomains [59] allowing peroxiredoxins to perform both signal transduction and peroxide scavenging roles. Note that in this model, thioredoxin oxidation during the peroxiredoxin catalytic cycle could also be considered a redox transduction event (see for example [109]). How does the signal peroxide model reconcile the over-oxidation properties of peroxiredoxins? In this model, peroxiredoxin overoxidation could release thioredoxin from peroxide catalysis to support other redox stress mechanisms [110], modulate peroxiredoxin-protein disulfide exchange and allow peroxiredoxins to oligomerize into chaperones to support the antioxidant response [87,111] including apoptosis for cells exposed to high levels of oxidative stress [112].

A number of bacterial transcription factors directly sense hydrogen peroxide and transduce a signal to the transcription machinery. For example, OxyR is a tetramer found primarily in Gram-negative but also in some Gram-positive bacteria [31]. In the presence of hydrogen peroxide, the peroxidatic or sensing cysteine of OxyR is rapidly oxidized ($\sim 10^5\ M^{-1}\ s^{-1}$) to a sulfenic acid which condenses to a disulfide bond, triggering a conformational change that allows the transcription factor to bind to its target DNA sequences [113,114]. The *oxyR* regulon includes genes involved in hydrogen peroxide detoxification, reductant supply and the repression of iron transport [115–117]. Oxidized OxyR is reduced by the glutathione/glutaredoxin system, reversing the conformational change and its binding properties [114,118]. A second factor, PerR, is a transcriptional repressor that is found mainly but not exclusively in Gram-positive bacteria and has metal co-factors in its binding and regulatory domains [31,119,120]. Once oxidized by hydrogen peroxide, PerR undergoes a conformational switch and can no longer repress transcription [31]. Similarly, OhrR is a dimeric repressor with a sensing cysteine that, upon oxidation by organic peroxides or sodium hypochlorite, forms a disulfide bridge that reduces the affinity of the transcription factor for its binding sequences [31].

3. Considerations for developing a quantitative redox signaling framework

Before reviewing and proposing methods available for quantifying redox signals, it may be worth considering the criteria that any proposed analytical framework should fulfil. While many different processes from thiol-disulfide exchange to hydrogen peroxide production have been loosely termed as redox signaling events, the exact nature of the redox signal has to our knowledge not been made explicit. As a result, it is not readily apparent which redox species or transduction event even constitutes the redox signal, and consequently quantitative measures of redox signaling have been wanting. Before developing a quantitative framework, the first requirement therefore is a qualitative working definition of redox signaling that provides a clear target or targets to measure. Secondly, as redox signaling appears to involve diverse redox

processes, any quantitative redox signaling measures should be flexible enough to both measure and compare these signaling regimes. Finally, the data needed to determine the quantitative redox signaling measures should ideally be experimentally tractable, although this is less of a concern as redox methodologies continue to evolve.

4. A qualitative definition of redox signal transduction

From the emerging principles described above, it is clear that redox-dependent thiol-signaling depends on changes in the hydrogen peroxide concentration, the kinetics of the reactants and the subsequent transduction of redox changes to other systems. In addition, as other oxidants such as superoxide can undergo conversions to form hydrogen peroxide [121], we propose a working definition for redox signaling as "a kinetic process involving coordinated changes in the oxidation state of redox transduction machinery leading to specific outputs in response to hydrogen peroxide and other oxidants." In this definition, a change in hydrogen peroxide levels is in itself not considered a signaling event unless it is accompanied by coordinated changes in the redox signal transduction machinery, and therefore the low levels of hydrogen peroxide produced during metabolism would not necessarily constitute a redox signal. Similarly, many of the non-specific oxidation events that result from severe oxidative insults would also not be considered true redox signaling events but part of a general stress response. On the other hand, the oscillatory changes in the peroxiredoxin oxidation state during circadian rhythms could constitute a genuine redox signal even though the physiological relevance of these oscillations is still being established [96,122]. Thus, an advantage of this definition is that it clearly distinguishes between redox signaling and non-specific oxidation events. Further, based on this definition and the accepted principles for redox signaling described above, an additional principle for redox signaling can be proposed. Like phosphokinase signaling, redox signaling appears to involve moiety-conserved cycles undergoing inter-conversions; it is therefore a molecular information transfer process driven by changes in oxidation states and not a bulk mass transfer metabolic process [32]. As such, the dynamic changes in the oxidation states of the redox transduction and output components and not the hydrogen peroxide concentration constitute the redox signal, and these changes could be used to quantify redox signaling.

A number of potential hydrogen peroxide-dependent redox transduction mechanisms have been described in the literature which have been classified into six schemes depending on the relationship between hydrogen peroxide and the target and sensor proteins [25]. However, in light of the definition above and based on the kinetic linkages between hydrogen peroxide and the target output, we have assigned these mechanisms into three broader, but not mutually exclusive categories: 'direct activation', 'sensor-mediated' and 'secondary' redox signaling (Fig. 2) which are analogous to the mechanisms recently proposed by Netto and Antunes [123]. In direct activation, a target-sensor protein is directly oxidized by hydrogen peroxide leading to a specific output. Here, the dynamics of the activation process depends primarily on the redox kinetics of this target protein whose redox state can be used to quantify the redox signal. The activation of *Escherichia coli* OxyR is the best characterized example of a directly activated redox signaling process (Fig. 2A) [113,114]. The conformational and activity changes in peroxiredoxins during sustained hydrogen peroxide exposure could also be regarded the output of a directly activated process as hyperoxidized, glutathionylated or disulfide-linked peroxiredoxins can themselves modulate other signaling systems. For example, it was shown that cell cycle arrest was correlated with peroxiredoxin 2 hyperoxidation and the formation of high molecular weight oligomers in mouse C10 lung epithelial cells [97]. Similarly, during infection and inflammatory processes peroxiredoxins can act as extracellular pathogen-associated molecular pattern (PAMP) and damage associated molecular pattern (DAMP) molecules [124–128] and it has been shown that

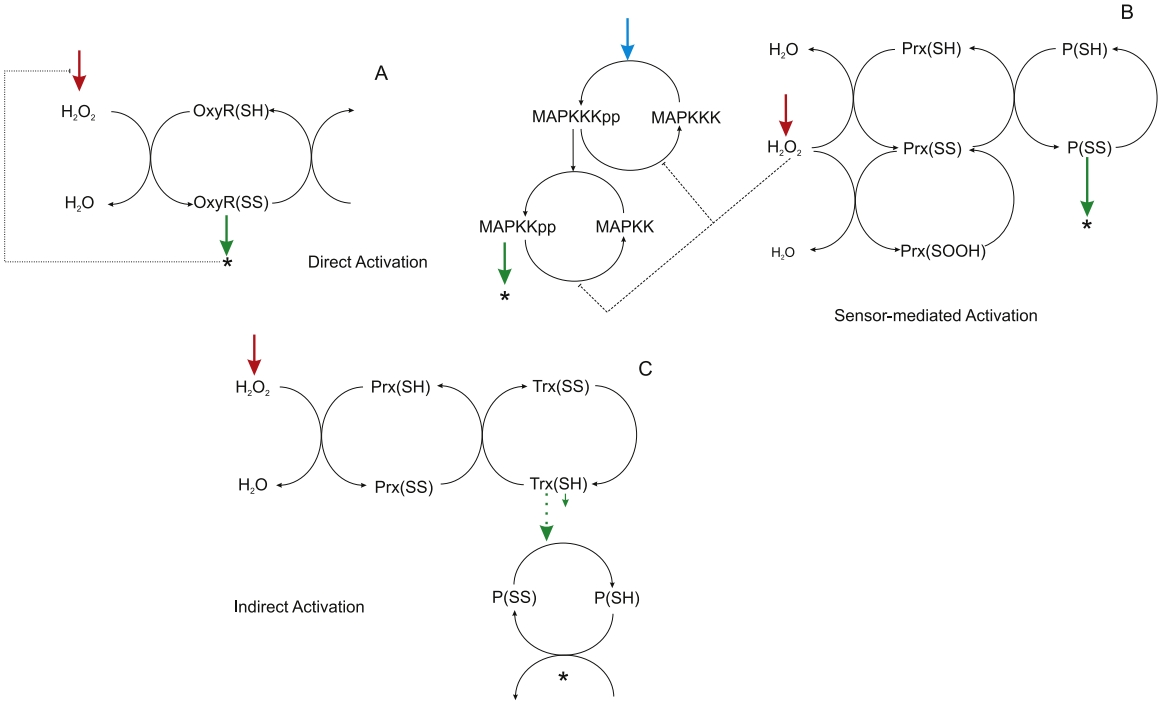

Fig. 2. Modes of redox signal transduction by hydrogen peroxide. For direct activation (A), the effect of hydrogen peroxide input (red arrow) on the output (green arrow) of a sensor protein (*e.g.* OxyR) depends primarily on sensor protein redox kinetics. In sensor-mediated activation (B), the effect of a hydrogen peroxide input can depend on other system inputs (blue arrow) and on the redox kinetics of steps linking hydrogen peroxide (red arrow) to the relevant signal outputs (green arrows). Shown in (C) is secondary activation in which hydrogen peroxide-mediated oxidation of thioredoxin decreases its availability for other pathways (details in text).

glutathionylated peroxiredoxin dimer release from macrophages and other cells acts as a 'danger signal' following lipopolysaccharide or TNFα stimulation [129–131].

With sensor-mediated signaling, hydrogen peroxide preferentially activates a sensor, which *via* redox cascades results in the activation or inactivation of a specific process. In contrast to direct activation, the dynamics of this signaling mode are a function of the kinetics of all the components in the transduction process and not just the sensor redox kinetics. An example of sensor-mediated activation would be the activation of phosphokinase signaling by the floodgate or peroxide signaling processes (Fig. 2B). This type of signaling exhibits two notable features. First, the output targets for this process, in this case the signaling kinases, usually react relatively slowly with hydrogen peroxide, and second, the flux through the kinase signaling pathway depends on both the mitogen and hydrogen peroxide concentrations (see for example [14,103,132–134]). Thus, signal transduction by this system could be considered as an "AND" circuit in terms of the Boolean logic [1] and the effect of both input signals have to be considered during quantification. Secondary redox signaling is a consequence of other signaling events that result in the oxidation of the thioredoxin and/or glutaredoxin redox couples, affecting their partner redox reactions (Fig. 2C). For example, peroxiredoxin-dependent oxidation of thioredoxin can reduce the flux to other thioredoxin-dependent reactions [135] and in the fission yeast, peroxiredoxin over-oxidation is a mechanism to restore the flux to these reactions [110]. Here, the transmission of the redox signal depends on the sensitivity of these partner reactions to changes in the concentration of their cognate redoxin (Fig. 2C) or more precisely their elasticities [136,137].

5. Analytical frameworks for quantifying redox signaling

The modes of signaling described above highlight key similarities with the better studied phosphokinase signaling systems and suggest how redox signaling could be quantified. Both redox and phosphokinase signaling appear to utilize moiety-conserved cycles to transfer molecular information, and mathematical models suggest that their respective interconnections can result in ultrasensitive switch-like behaviors, an established property of signaling systems [4,135]. However, while phosphokinase signaling usually results in unidirectional chains of activation from an external mitogen (Fig. 1), hydrogen peroxide could potentially induce direct, sensor-mediated or secondary redox transduction processes simultaneously (Fig. 2), complicating the analysis of these systems. Nonetheless, in common with the analysis of MAPK signaling [2,5] or sensory perception [138] systems, redox signaling could be analyzed in terms of time and concentration as it involves both temporal and steady state changes to the redox signaling machinery (see for example [104,113,132,139]). A number of approaches have been developed to quantify signaling and these will be described in the context of redox signaling below.

5.1. Time-dependent signaling parameters

Heinrich and colleagues developed a set of signaling parameters for characterizing the time-dependent signal properties produced during protein kinase signaling [2]. In this framework, the total amount of target product (P_i) produced over a signaling interval (t) is used to determine the signaling time (τ_i), signal duration (ϑ_i) and signal amplitude (S_i), which describe the average time to activate a target protein, the average time that the target protein is active, and the average concentration of active target protein over the signaling interval, respectively [2,140]. In order to determine these parameters, the total amount of activated target protein (I_i) is calculated from the area under the curve of activated target protein (P_i) against time (Fig. 3 B, [2,140]):

$$I_i = \int_0^\infty P_i(t)\,dt \tag{1}$$

The signaling time is an average described by:

$$\tau_i = \frac{T_i}{I_i} \tag{2}$$

where

$$T_i = \int_0^\infty t{\cdot}P_i(t)\,dt \tag{3}$$

The signal duration (ϑ_i) is determined analogously to a standard deviation around the average time (Eq. (4)) and is also used to calculate the signal amplitude (S_i, Eq. (5)). The signal amplitude is not necessarily the maximum amplitude obtained during the signaling interval (Fig. 3B) but represents the height of a rectangle of length $2\vartheta_i$ whose area is equal to the area under the curve [2].

$$\vartheta = \sqrt{\frac{\int_0^\infty t^2 P_i(t)\,dt}{I_i} - \tau_i^2} \tag{4}$$

$$S_i = \frac{I_i}{2\vartheta_i} \tag{5}$$

Practically, these measures can be readily determined using mathematical software once I_i is obtained. Therefore, a major advantage of this approach is that virtually any time-dependent redox signaling regime can be quantitatively compared against other signaling regimes allowing for comparisons between cell states, tissues and organisms. By way of example, we developed a core computational model based on the kinetics for OxyR activation and reduction (Fig. 3A). The model was simulated with two different glutathione reductase concentrations and the resulting signal parameters could be used to quantitatively describe the role of glutathione reductase in the OxyR response (Fig. 3B). A full description of the model may be found in the supplementary information and we emphasize that this is only a core model [66] which was not fitted to an *in vivo* dataset. These measures can also be used to deduce the signal control properties within a reaction network, which can provide insights into functional organization of these systems (readers are referred to [3,136,140] for a description of control analysis).

A potential limitation with this approach is that the choice of target protein is subjective as simulation results on a phosphokinase signaling cascade have shown that the different cycles within the same signaling cascade can have distinct signal parameters [140]. Thus, caution must be exercised in the choice of target protein(s) which may not be readily apparent, especially in sensor-mediated and secondary redox signaling processes (Fig. 2). An additional caution with this method relates to the calculation of I_i, which can be estimated from western blotting data (see [139] for an example of a time-dependent protein activation curve from western blotting data). As I_i is estimated from the area under the target protein curve (Fig. 3B), multiple time points would significantly improve the accuracy of the signal parameter estimates obtained. Nonetheless, it would probably still be necessary to fit these data to kinetic models or mathematical functions, which is not a trivial problem given the uncertainty about the kinetic models for peroxiredoxin activity (see below).

5.2. The effect of input concentrations on the redox signaling response

In addition to time, redox signaling is also sensitive to the

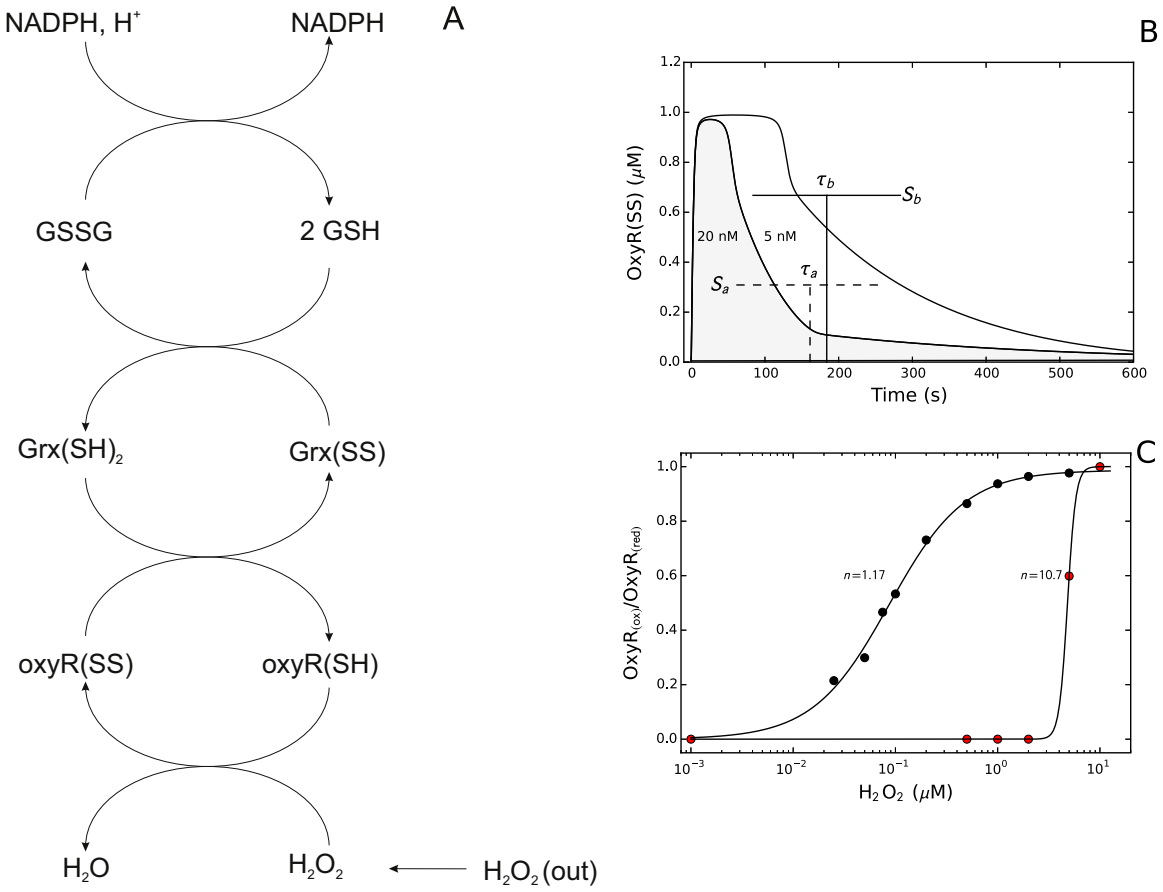

Fig. 3. Time and concentration dependent dynamics for OxyR activation. A kinetic model of the activation of *E. coli* OxyR by hydrogen peroxide (A) was developed using the Python Simulator for Cellular Systems (PySCeS) [191]. (B) The effect of bolus hydrogen peroxide (100 μM) on the time-dependent signal parameters obtained from the kinetic model (Supplementary information) was determined at glutathione reductase concentrations of 5 nM (a, white fill) and 20 nM (b, grey fill). The signaling parameters for OxyR activation for a glutathione reductase concentration of 20 nM were: $I_a=124.42$, $\tau_a=161.15$, $\vartheta_a=201.43$ and $S_a=0.31$ while the signal parameters at 5 nM glutathione reductase were $I_b=250.96$, $\tau_b=183.82$, $\vartheta_b=187.97$ and $S_b=0.67$. Shown on the figure are the signaling times and amplitude for OxyR activation in these two conditions (details in text). In (C), OxyR activation as a fraction of the total OxyR concentration was quantified from *in vitro* (black) and *in vivo* (red) data [113] and fitted to the Hill equation by non-linear regression analysis. This analysis yielded Hill coefficients of 1.17 and 10.7 for the *in vitro* and *in vivo* activation of OxyR respectively. Further details including Hill plots for these data are provided in Supplementary information.

prevailing hydrogen peroxide concentrations; this effect has been described in numerous studies and with several different experimental approaches. These studies include determining the bimodal responses to hydrogen peroxide concentrations (see for example [9,141–143]), defining hydrogen peroxide toxicity levels [144,145], quantifying the transcriptional response to increasing hydrogen peroxide concentrations [117,146], measuring the hydrogen peroxide concentrations needed to activate a transcription factor or affect a signaling process [113,132,147,148], as well as estimating the hydrogen peroxide gradient across a cell membrane [149,150]. Collectively, these approaches have stimulated the development of hydrogen peroxide delivery methods to increase the reproducibility and physiological relevance of these experiments [151–153] and have provided quantitative information on the concentration-dependent effects of hydrogen peroxide in a number of cellular processes. In terms of redox signaling, recent work by Brito and Antunes [154] has extended previous studies on hydrogen peroxide gradients across cell membranes [149] to provide estimates for the rate constants for the hydrogen peroxide-dependent activation and deactivation of signaling targets.

A number of studies on the effect of input concentrations on signaling have also uncovered interesting properties about the architecture of the transduction systems themselves that may be relevant to redox signaling. For example, early theoretical modeling studies on phosphokinase signal transduction cascades predicted that changes in mitogen concentration could trigger ultrasensitive changes in the terminal kinase concentrations in these cascades [4]. These sigmoidal or switch-like responses were subsequently shown to be a critical feature of many biological processes such as the all-or-none maturation response in frog oocytes [155], embryonic patterning in *Drosophila* [156] and mating decisions in *Saccharomyces cerevisiae* [157]. Other signaling cascade properties that have been described using this approach include kinetic proof-reading [158], signal adaptation [159] and noise attenuation [160].

Does the architecture of redox signaling systems allow for these properties? Computational modeling of the *E. coli* redoxin network revealed that the reduced thioredoxin concentration can indeed undergo ultrasensitive changes *via* an unusual mechanism that was dependent on the rate constants for peroxiredoxin reduction and oxidation [135]. OxyR activation has shown cooperative behavior *in vitro* [161,162] and we reanalyzed published data on *E. coli* OxyR activation *in vivo* [113] and found evidence of ultrasensitive activation of OxyR in response to increasing hydrogen peroxide concentrations (Fig. 3C). An ultrasensitive, rather than a Michaelis-Menten-like hyperbolic response, would allow the bacterium to fully induce transcription of the *oxyR* regulon over a much narrower range of hydrogen peroxide concentrations, facilitating a more sensitive response to increasing and potentially toxic levels of this oxidant [43,145]. We also noted that the Hill coefficient for ultrasensitive response *in vivo* ($n=10.7$) was greater than the coefficients reported *in vitro* ($n=1.17-3.5$) (Fig. 3C,

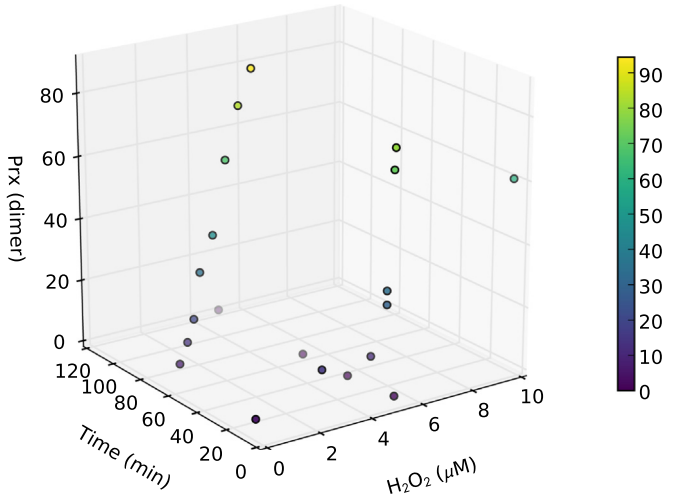

Fig. 4. Peroxiredoxin-2 dimerization is a function of time and hydrogen peroxide concentration in HEK293 cells. Redox western blotting was used to quantify peroxiredoxin-2 dimerization in HEK293 cells but at high hydrogen peroxide concentrations peroxiredoxin-2 became overoxidized and migrated as a monomer and some of these data points are not shown on the figure (data taken from Fig. 6A-C, E in Ref. [152]).

[161,162]) suggesting that cooperative hydrogen peroxide binding by the oxyR tetramer is only partially responsible for the ultrasensitive response *in vivo*. Curiously the kinetic linkages involved in OxyR activation (Fig. 3A, [114]) do not resemble any previously described ultrasensitive mechanisms [5], implying that redox signaling may utilize previously uncharacterized motifs to achieve similar signaling outcomes to phosphokinase signaling cascades.

5.3. Time and concentration dependent effects

As redox signaling involves both time and concentration dependencies, an interesting question is whether these factors have synergistic effects. To the best of our knowledge this has not been systematically tested and as a first step we reanalyzed the time and concentration data presented by Sobotta et al. [152] (Fig. 4). In these experiments HEK293 cells were exposed to bolus hydrogen peroxide concentrations over different times and the fraction of dimeric (oxidized) peroxiredoxin-2 was quantified by redox western blotting. A limitation with this analysis was that at long exposure times and/or high hydrogen peroxide concentrations, peroxiredoxin-2 became hyperoxidized and migrated at a similar size to the reduced peroxiredoxin-2 monomer [152]. Nonetheless, the data indicated that exposure time and hydrogen peroxide concentration are indeed related, as reducing exposure time and/or lowering the hydrogen peroxide concentration reduced the formation of the dimeric and hyperoxidized peroxiredoxin-2 forms. While we have insufficient data to populate this data space completely (Fig. 4), these results suggest that there may be regions where peroxiredoxin over-oxidation is minimized. Whether redox signaling events such as the oxidation of transcription factors are supported within these regions remains an open and interesting question.

How cells balance these sometimes competing factors of exposure time, hydrogen peroxide concentration and peroxiredoxin activity with effective signaling, could be cast as an optimization problem [163]. Optimization is a broad field of applied mathematics with applications in economics, engineering, biology, bioinformatics and systems biology. Briefly, optimization involves the use of mathematical tools to determine the best combination of decision variables to maximize or minimize an objective function. In the case of redox signaling, the decision variables could be hydrogen peroxide concentration and exposure time and the objective function could be the maximization of transcription factor activation and the minimization of oxidative stress as determined by peroxiredoxin hyperoxidation. The concentrations and kinetics of the components involved in redox signaling represent system constraints and are also included in the optimization problem. This analysis could yield important quantitative insights into redox signaling but perhaps more importantly, could also be used to infer the objective functions used for redox signaling (see for example [164]).

5.4. Supply-demand analysis

Supply-demand analysis is a powerful framework to quantify the control of flux and the metabolite concentrations within cellular networks [165–167]. Unlike the standard textbook descriptions of metabolic regulation, this framework considers the effect of metabolite demand on the regulatory properties of metabolic biosynthetic pathways. In this framework, a metabolic pathway or indeed several pathways are aggregated into a 'supply' block that produces a linking intermediate, which is consumed by a 'demand' block that could also represent a number of metabolic pathways (*e.g.* an amino acid biosynthesis pathway could be considered a supply block producing an amino acid which is then used by anabolic demand pathways). An important insight from supply-demand analysis was that the function of allosteric regulation in many biosynthetic pathways is not flux control as was commonly assumed, but to maintain metabolite concentrations within homeostatic levels [167]. In this method, the supply and demand rate characteristics (*i.e.* the variation of the supply and demand rates with changing intermediate concentrations) are plotted in double-logarithmic space and the intersection of these curves represents the steady state of the system. A major advantage of this method is that the steady-state supply and demand elasticity and flux-control coefficients can be calculated, and the rate characteristic plot also provides an intuitive and visual representation of the regulatory profile of the system [166].

In terms of redox signaling, the production of hydrogen peroxide and its consumption by the cellular antioxidant defense network could be aggregated into supply and demand blocks respectively. To illustrate this method, kinetic models of the *E. coli* thiol peroxidase Tpx system [135], the AhpC [168,169] and catalase [170] enzymes were used to simulate hydrogen peroxide demand rate characteristics (Fig. 5). To model the supply rate characteristics, endogenous hydrogen peroxide production rates above and below 14 $\mu M\, s^{-1}$ [168] were used, as intracellular hydrogen peroxide production in *E. coli* depends on the number of active respiratory chains and is therefore affected by the growth phase of the cell [171]. In this simulation, AhpC and catalase were modeled with Michaelis-Menten kinetics [169,170] and hydrogen peroxide production was modeled as a constant supply assuming that the intracellular hydrogen peroxide concentration did not cause product inhibition or significant respiratory chain oxidation [172]. The intersection of the supply and demand rate characteristics with hydrogen peroxide production rates up to 14 $\mu M\, s^{-1}$ revealed steady-state intracellular hydrogen peroxide concentrations that were less than 0.1 μM (dashed lines, Fig. 5) in agreement with experimental results [173]. With a higher hydrogen peroxide supply, the steady hydrogen peroxide levels were in the range of $0.1-1$ μM (shaded regions, Fig. 5) which could lead to increased mutagenesis and growth arrest *in vivo* [168,174], although induction of antioxidant gene expression could alter the demand rate characteristics to match this supply.

A limitation with the analysis described here was that we used assigned Tpx, catalase and AhpC concentrations and parameters such as NAD(P)H were considered fixed within the model.

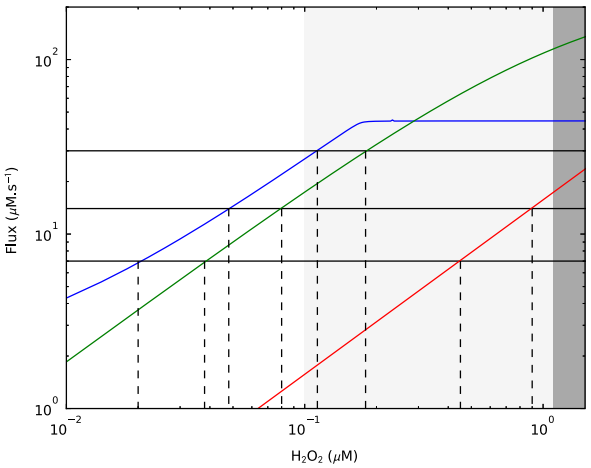

Fig. 5. Supply-demand curves for hydrogen peroxide consumption in a computational model of hydrogen peroxide metabolism in *E. coli*. In this analysis a constant metabolic supply of hydrogen peroxide (black lines) at 7, 14 [168] and 30 μM s^{-1} was assumed and hydrogen peroxide demand by the *E. coli* Tpx and thioredoxin system (blue) [135], AhpC (green) and catalase (red) were plotted independently. The intersections of the supply and demand curves reveal the steady-state flux and hydrogen peroxide concentrations in the model with the light grey and dark grey shading representing hydrogen peroxide concentrations that can trigger mutagenesis or growth arrest. The Tpx rate characteristic has been described previously [135] and the parameters used for AhpC and catalase were k_{cat}=52.2 s^{-1}, K_m=1.4 μM [169] and k_{cat}=11,000 s^{-1}, K_m=3.5 mM [170] respectively.

Nonetheless, this analysis revealed an interesting feature of hydrogen peroxide metabolism in *E. coli*, which provides an additional answer to the excellent question raised by Mishra and Imlay as to why bacteria have so many hydrogen peroxide degrading activities [30]. The supply-demand plot shows that these activities can act synergistically in the face of increasing hydrogen peroxide supply, to limit the steady state hydrogen peroxide concentration (Fig. 5). By contrast, with changes in hydrogen peroxide supply, the steady state hydrogen peroxide concentration could change significantly if these demand activities were operating in isolation. For example, changes in the hydrogen peroxide supply can change the intracellular steady state hydrogen peroxide concentration from 20 nM to 0.113 μM if AhpC was acting alone, but in combination with Tpx, a number of stable steady states can be established at lower hydrogen peroxide concentrations in our simulations (Fig. 5). This analysis also highlighted a previously unappreciated advantage of peroxiredoxin systems. If the kinetic profile of peroxiredoxins could be altered by changes in the activity or concentration of their cognate reductants and/or reductases, the steady state intracellular hydrogen peroxide levels [173] could be tuned to different supply regimes by adjusting the levels of the components of these systems. Thus, supply-demand analysis can provide a global understanding of kinetic regulation even within a large network of reactions by considering this network in context of the cellular environment.

Experimentally, supply-demand curves are generated using a double modulation method in which changes to the supply block are used to modulate the intracellular concentration of the linking metabolite, allowing the rate characteristic plot of the demand block to be determined. Similarly, changes in the demand block are used to determine the rate characteristic plot of the supply block (reviewed in [166]). This method therefore depends on the ability to modulate and determine the fluxes of both the supply and demand blocks and to determine the intracellular concentration of the linking metabolite. The use of genetically encoded redox probes has now made it possible to accurately determine the intracellular hydrogen peroxide concentrations (see for example [150]) and it is also possible to modulate the intracellular hydrogen peroxide supply by exogenously added hydrogen peroxide [151–153]. However, it is not clear whether hydrogen peroxide demand activities can be experimentally modulated over a sufficiently large dynamic range to ensure that the supply rate characteristic plot is accurately determined. In addition, most cells have multiple routes for hydrogen peroxide reduction and determining the fluxes through all of these pathways may not be experimentally tractable. A further consideration with this method is that it depends on the supply and demand blocks communicating solely by the linking metabolite [167]. At timescales larger than minutes, transcription of oxidative stress induced genes could effectively change the activities within the demand block complicating these experiments. Nonetheless, the ever increasing development of detailed computational models of redox systems (see for example [66,135,175–178]) offers another avenue for the application of supply-demand analysis. In the original iteration of the method [167], the supply and demand blocks were selected based on existing knowledge of the system but in generalized supply-demand analysis each variable species in a computational model is fixed and modulated, generating supply-demand rate characteristics across all reactions in the entire model [179]. An advantage of this approach is that it could be used to identify kinetic motifs relevant for redox signaling that may only be apparent once the complete network of reactions are analyzed.

6. Challenges and limitations

The common and arguably the most significant limitation with all the quantitative frameworks described above, is the requirement to have datasets that are sufficiently comprehensive for accurate analysis. As redox signaling can potentially involve multiple cellular processes (see for example Fig. 2B), obtaining such datasets will be challenging even with the availability of high-throughput and multi-omics technologies [8,180–184]. In metabolic studies, computational modeling has been central to the exploitation and analysis of omics datasets [185] and could therefore serve as a powerful complementary tool in redox signaling studies [66]. For example, computational modeling techniques could be used to simulate missing data, inform experimental strategies by delineating parameter ranges that support redox signaling, or could also be used to identify motifs [186] that effect redox signaling.

There are, however, some considerations that could limit the application of computational modeling in such studies. The construction of realistic computational models depends on accurate kinetic parameters, but in many instances these parameters are unavailable, even for model organisms. There has also been uncertainty on the choice of kinetic expressions to describe thioredoxin, glutaredoxin and peroxiredoxin (redoxin) activity in computational systems biology models [66]. While kinetic expressions for the thioredoxin [187,188] and glutaredoxin [189] systems appear to be resolved, the appropriate kinetic expression (s) for modeling peroxiredoxin activity has not been settled. The consensus within the field is that peroxiredoxins are peroxidase enzymes with (bi-bi) ping-pong kinetics [190], but peroxiredoxins have been modeled as reactants with mass action kinetics in all computational models of the system published thus far [135,175,176]. Even within these models, peroxiredoxins have been treated either as monomers (*e.g.* [135]) or as dimers (*e.g.* [175]) where each peroxidatic cysteine was modeled explicitly (Fig. 6). In computational models of such systems these peroxiredoxin kinetic expressions will not be interchangeable and will result in models with distinct steady-state rate and concentration properties. As peroxiredoxins are key mediators of redox signaling, determining the appropriate kinetic expression for peroxiredoxin

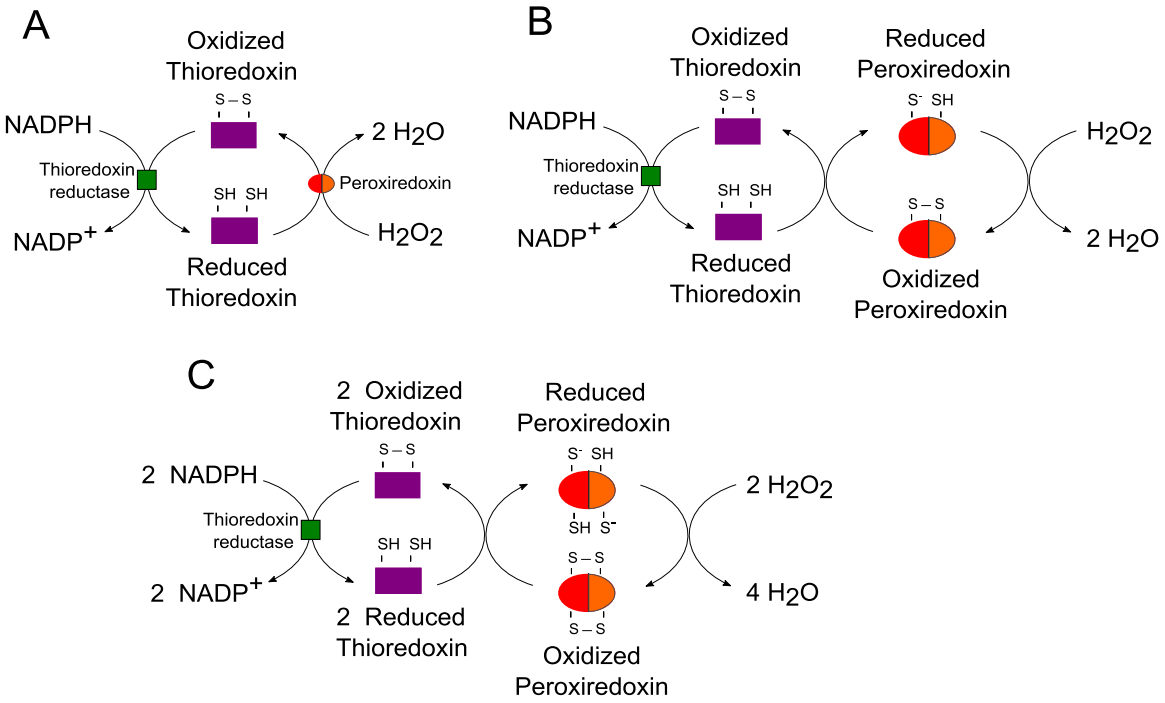

Fig. 6. Distinct kinetic models have been proposed for peroxiredoxin activity. Peroxiredoxins are widely considered to be (A) ping-pong enzymes, although peroxiredoxins have been described as (B) monomers or as (C) homodimers with mass-action kinetics in computational models (see text for details). Note that stoichiometry of hydrogen peroxide reduction is different in the monomer and homodimer kinetic models of peroxiredoxin activity.

activity is a critically important question confronting computational modeling efforts.

A further potential limitation with the approaches described here is that quantification is focused on the output(s) of a given redox signaling process and not necessarily on the chain of molecular events leading to redox signaling. For example, in sensor-mediated signaling a hydrogen peroxide stimulus can affect multiple components including other signaling systems but the frameworks described above focus primarily on the outputs of the signaling process for quantification (green arrows, Fig. 2B). This simplification is however balanced by a number of significant advantages. First, this 'global' approach to signaling can lead to new insights or experimental directions as shown by our analysis of OxyR activation *in vitro* and *in vivo* (Fig. 3C). Second, provided that the system under study is reasonably well-characterized, this approach can reduce the experimental effort needed to quantify redox signaling in a system. Finally and as described above, these quantitative frameworks can allow for comparisons of even distinct redox signal transduction systems which may yield insights into the type of relationships that cells in different niches have with hydrogen peroxide.

7. Final remarks

There has been a growing appreciation that quantitative approaches can provide valuable insights into redox regulation for both basic and clinical research applications [62,66]. An implicit advantage of these approaches is that they require precise descriptions of the components and processes within a system, forcing investigators to critically interrogate the meaning and values attached to them. In this paper we reviewed the oxidant, targets and systems involved in redox signaling. We then proposed definitions for redox signaling and the redox signal itself which allowed us to focus on a number of quantitative frameworks for analyzing such signaling. While each of these frameworks has its own strengths and limitations, they all depend on extensive datasets for accurate analysis and computational modeling may be an important complementary tool for these analyses. Going forward these frameworks will allow us to better understand, quantify and compare redox signaling which is expected to yield insights into redox biology, pathologies associated with dysfunctional redox signaling and may lead to the rational design of redox circuits for biotechnological applications.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.04.199.

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